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13. ABSTRACT (Maximum 200 Words)

New chemotherapeutic agents are needed for the improved treatment of breast cancer. In this proposal, we disclose a new approach to the design of anti-cancer drugs. Our method is to synthesize new drug conjugates that incorporate: (i) a specific breast cancer cell -targeting component; (ii) a rapid cell membrane translocating /nuclear localization moiety and; (iii) the capability to counter multi-drug resistance mediated by P-glycoprotein and related cellular efflux pumps. The conjugates are prepared in a few synthetic steps from available components. The goal is to demonstrate a proof-of-concept of the effectiveness of this multi-functional drug hypothesis.

Specific cancer cell-targeted compounds have been prepared from the breast cancer drug doxorubicin. This cytotoxic agent is covalently linked to a synthetic arginine-glycine-aspartic acid peptidomimetic, which recognizes and binds to a_vb_3 integrin. This receptor is overexpressed on the surface of breast cancer metastatic cells and tumors. The design also includes incorporation of the Tat peptide analog, H_2N[arginine]$_7$COOH, as a rapid cell membrane translocation and effective nuclear localization moiety. The new drugs will be evaluated in breast cancer cell-lines *in vitro* and *in vivo* using human breast cancer xenografts in nude mice.

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Table of Contents

A. Introduction

In this program, we are examining a new approach to the design of anti-cancer drugs that is directed toward (i) improving cytotoxic action against cancer cells, (ii) reducing unwanted systemic side effects, (iii) counteracting multi-drug resistance, and (iv) targeting and destroying metastatic cells as well as tumors more effectively.

Our plan is to synthesize new drug conjugates that incorporate a specific breast cancer cell targeting component, a rapid cell membrane translocating/nuclear localization moiety, and the capability to counter multi-drug resistance mediated by P-glycoprotein and related cellular efflux pumps. The conjugates will be prepared in a few synthetic steps from available intermediates. The goal is to demonstrate a proof-of-concept of the effectiveness of this multi-functional drug hypothesis.

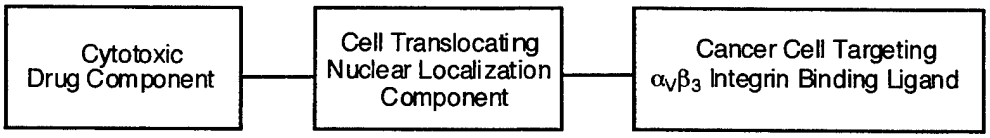
Specific cancer cell-targeted compounds are being prepared from the breast cancer drug doxorubicin. This cytotoxic agent is covalently linked to a synthetic arginine-glycine-aspartic acid peptidomimetic, which recognizes and binds to $\alpha_V\beta_3$ integrin overexpressed on the surface of breast cancer metastatic cells and tumors. The design also incorporates the Tat peptide analog, H_2N[arginine]$_7$COOH, as a rapid cell membrane translocation and effective nuclear localization moiety. Because the targeted conjugates will be rapidly directed into the cell nucleus for efficient cytotoxic effects, the drugs may escape cytoplasmic cleansing, which is mediated by cellular efflux pumps thereby abrogating an important multi-drug resistance mechanism. The new drugs will be evaluated in breast cancer cell-lines *in vitro* and *in vivo* using human breast cancer xenografts in nude mice.

B. Body

This section describes research accomplishments to date associated with the tasks outlined in the original award application.

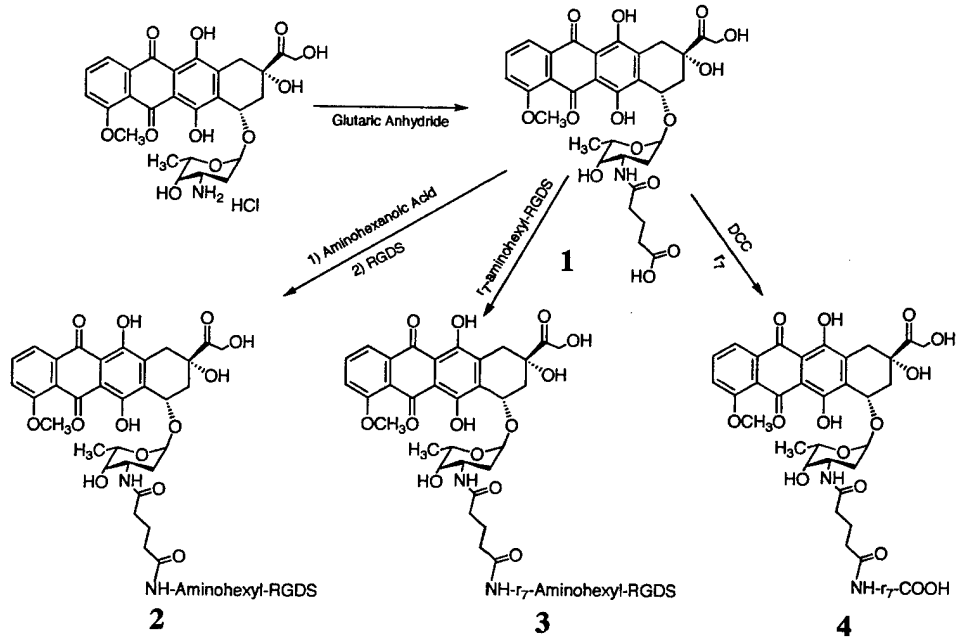
Task 1. Synthesize several covalent conjugates utilizing the anti-tumor drug doxorubicin, which are linked to a cell translocating/nuclear localizing arginine peptide and a selective breast cancer cell targeting ligand, as well as appropriately linked components as controls **(Months 1-18)**

The three-component conjugates are being assembled according to the arrangement shown below.

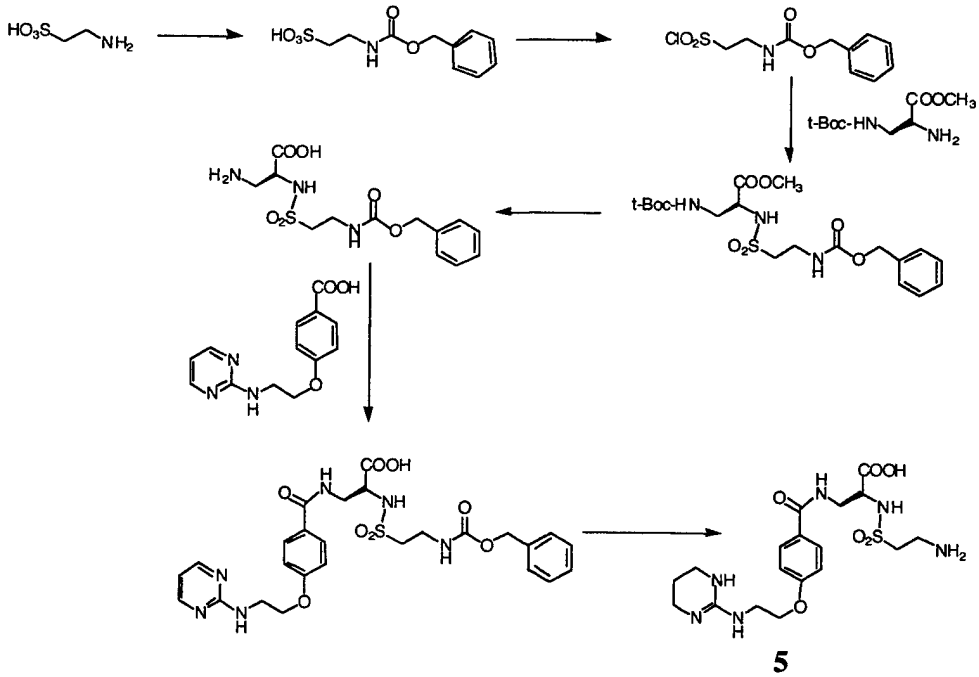


As reported in our first annual report last year, we examined unsuccessfully a number of approaches to prepare our proposed conjugates using doxorubicin derivatives substituted at C-14. We then turned our attention to doxorubicin conjugates derivatized at the sugar amine (**Scheme 1**). Derivatized doxorubicin **1** was condensed, after carbodiimide activation, with the cell-translocating peptide, H_2N[D-arginine]$_7$COOH (r_7) and with the peptide H_2N[D-arginine]$_7$CONH-Aminohexyl-RGDS (r_7-Aminohexyl-RGDS), which incorporates the relatively low affinity $\alpha_V\beta_3$ integrin peptide-ligand, arginine-glycine-aspartic acid-serine

(RGDS). In this way, we have successfully prepared conjugates **2**, **3**, and **4** and have begun preliminary cell localization experiments (*Task 2*) as well as cytotoxicity studies (*Task 3*). Conjugate **4** will be derivatized with the high affinity $\alpha_v\beta_3$ integrin ligand **5** (**Scheme 2**)[1]. Graduate Research Assistant, Jiang Sha, has now synthesized compound **5.**



Scheme 1

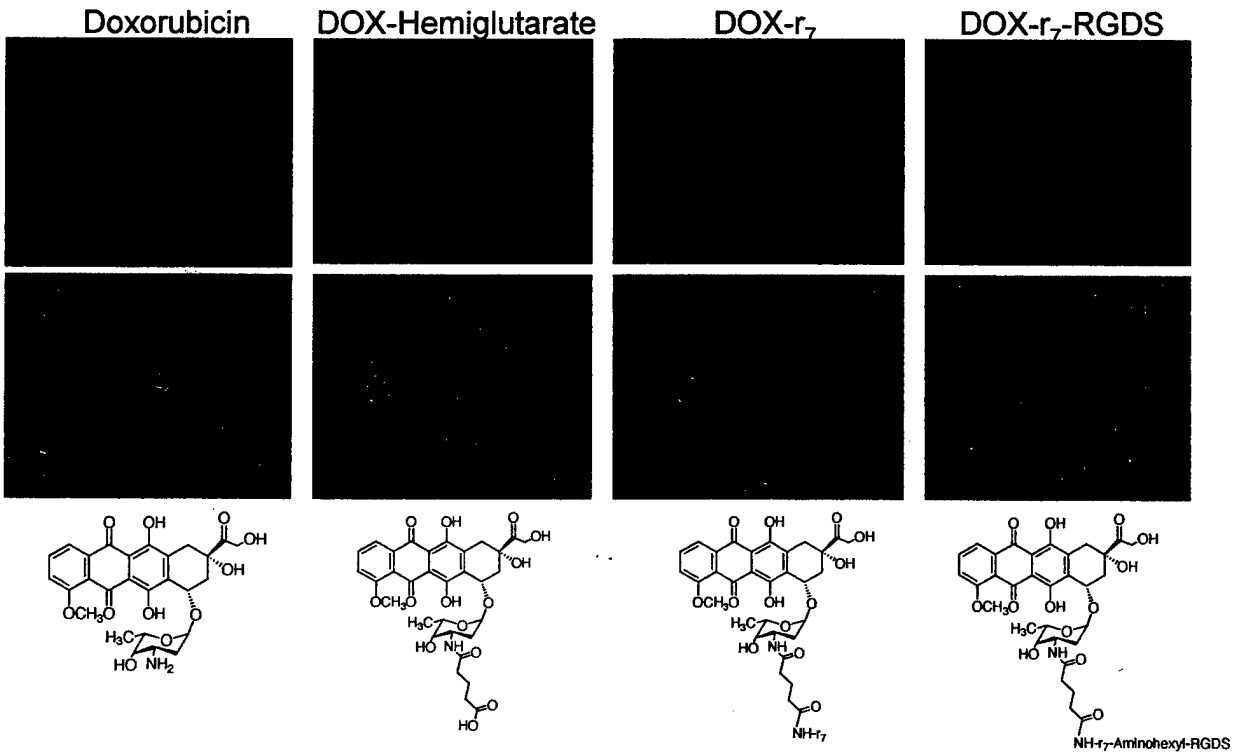


Scheme 2

Task 2. Establish analytical approaches (confocal microscopy) to monitor the translocation of the doxorubicin conjugates into cells **(Months 9-24)**

Using the inherent fluorescence of doxorubicin, we have followed the translocation of the conjugates synthesized in **Scheme 1** into cancer cells. **Figure 1** shows fluorescent micrographs (400X) of the uptake of the compounds into MDA-MB-231 breast cancer cells.

Figure 1. Fluorescence Microscopy, 50 μM Conjugate
30 min Incubation At 37°C

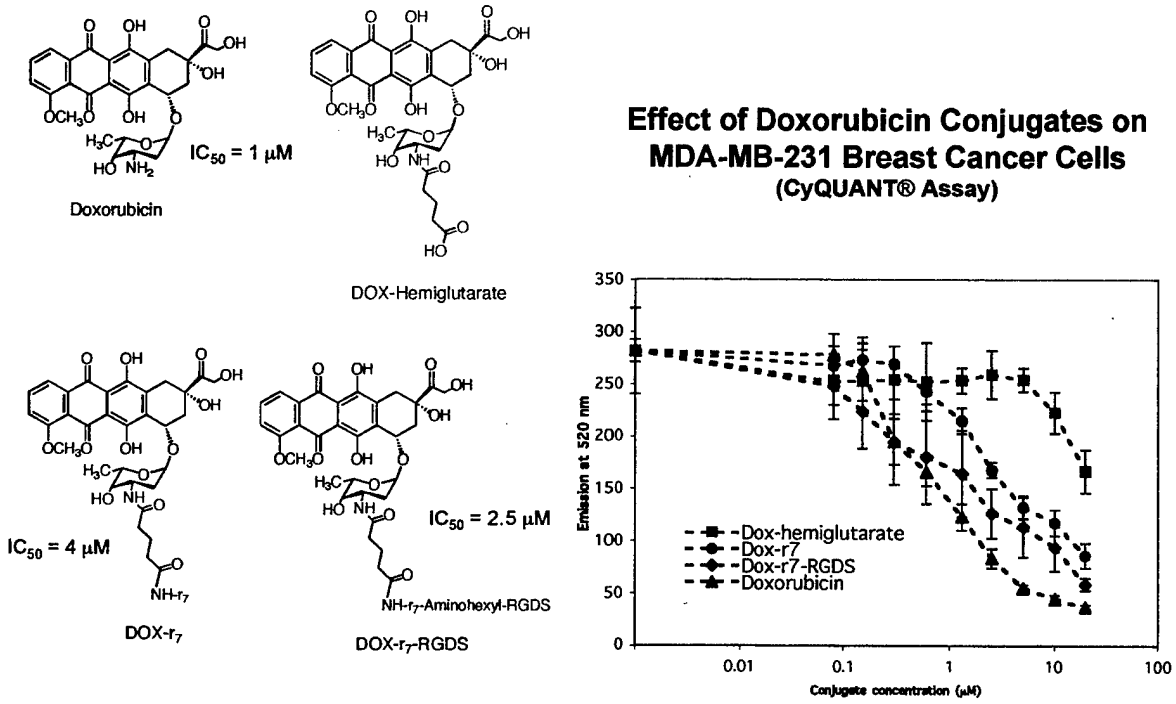


As is apparent from **Figure 1,** the parent drug, doxorubicin, is readily taken into the cells. As anticipated, DOX-hemiglutarate is not taken into the cells. This is consistent with earlier work, which has established that N-acylation of the amino sugar of doxorubicin often abrogates cell cytotoxicity[2]. On the other hand, attachment of either r_7 or r_7-RGDS promotes the cancer cell uptake of the conjugates. These results are in accordance with cell cytotoxicity results (see below).

Task 3. Compare the cytotoxic efficacy of the drug conjugates (*vs.* free doxorubicin) in human breast cancer and normal breast cell lines **(Months 12-24).**

The conjugates were evaluated for cytotoxicity against the $\alpha_v\beta_3$ expressing breast cancer cell-line MDA-MB-231 (**Figure 2**). After 24 hr exposure to the drugs, viable cells were assayed using the CyQuant™ cell proliferation assay (Molecular Probes, Eugene, OR). Most exciting about the results in **Figure 2** is that the activity of the DOX-r7-RGDS compound is comparable to DOX itself. This is interesting and promising because DOX derivatives acylated on the carbohydrate amino group often show considerably reduced toxicity[2] (e.g., note the lowered activity of DOX-Hemiglutarate in **Figure 2**). It appears that our cell translocating-targeting concept can even improve the activity of poorly performing DOX derivatives.

Figure 2. Cell Cytotoxicity



Task 4. Evaluate the efficacy of the conjugates (*vs.* free doxorubicin) in human breast cancer tumor xenografts in nude mice **(Months 24-36)**

This task is scheduled for later in the program.

C. Key Research Accomplishments

Key accomplishments from Year Two of this research are listed below.

Doxorubicin conjugates have been prepared incorporating the [D-arginine]$_7$ cell membrane translocating functionality.

A doxorubicin conjugate incorporating the [D-arginine]$_7$ cell membrane translocating group coupled to the low affinity $\alpha_v\beta_3$ integrin ligand, RGDS has been synthesized.

A high affinity $\alpha_v\beta_3$ ligand has been synthesized for coupling to doxorubicin-r_7.

All newly-synthesized compounds have been purified and chemically characterized.

Several conjugates have been evaluated for their effects on the breast cancer cell line MDA-MB-231.

Ongoing experiments are examining the translocation of the conjugates into cancer ous and non-cancerous cells.

D. Reportable Outcomes

This program supports graduate research assistant, Jiang Sha, and the results from the research will be incorporated into his dissertation. Postdoctoral research associate Hee-Kyoung Lee also assists in this effort. A manuscript is in preparation outlining the synthesis of the compounds prepared to date and their effects in breast cancer cell lines.

E. Conclusions

Research on this effort thus far has provided modified doxorubicin intermediates suitable for attachment to a cell membrane translocating functionality and $\alpha_v\beta_3$ integrin targeting ligands. The resulting conjugates are being evaluated breast cancer cell culture experiments in order to ascertain cytotoxicity as well as selectivity for cancer cells over normal cells.

This research is significant in that it represents the first known examples of cancer chemotherapeutic agents incorporating a drug chemically linked both to a breast cancer-targeting moiety as well as a cell membrane translocating/nuclear localization functionality. The conjugates are expected to show selective targeting to breast cancer cells in preference to normal cells as well as exhibiting enhanced cancer cell cytotoxic effects. Preliminary results reported here are beginning to support the promising nature of this idea.

F. References

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(2) Arcamone, F., Doxorubicin, In: Anticancer Antibiotics*; Academic Press: New York, 1981; Vol. 17.*

G. Appendix

Biosketches

Jerald C. Hinshaw, Principal Investigator

Jiang Sha, Graduate Research Assistant

BIOGRAPHICAL SKETCH	
NAME **HINSHAW, JERALD CLYDE**	POSITION TITLE Research Associate Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing. Include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
Oregon State University, Corvallis, Oregon	BS	1962 - 1966	Chemistry
The University of Utah, Salt Lake City, Utah	PhD	1966 - 1970	Organic Chemistry

Research and Professional Experience:

1970-1978 Advanced from Senior Research Chemist to Research Associate, Organic Research Laboratory, Chemistry Division, Research Laboratories, Eastman Kodak Company
1978-1984 Scientist, Research and Development Laboratories, Thiokol Corporation
1980, 1986 Member, Utah Award Committee, Salt Lake Section, American Chemical Society
1981 Visiting Research Associate, University of Utah.
1981-1983 Chairman-Elect, Chairman, Past-Chairman, Salt Lake Section, American Chemical Society
1984-1990 Supervisor, Propellant Research Section, Research and Development Laboratories, Thiokol Corporation
1990-1999 Manager, Energetic Materials Research Department, Research and Development Laboratories,Thiokol Propulsion, Brigham City, Utah.
1996-1999 Member, State Advisory Council on Science and Technology (State of Utah, Governor appointment)
1997,1998 Member, Utah State Governor's Medal for Excellence in Science and Technology Award Committee
1997-1999 Chairman, State Advisory Council on Science and Technology (State of Utah, Governor appointment)
1997-1999 Member, Utah Centers of Excellence Program Advisory Council (State of Utah, Governor appointment)
2/99-7/99 Senior Staff to the Technical Director, Science and Engineering, Thiokol Propulsion, Brigham City, Utah
7/99-11/01 Research Assistant Professor, Department of Medicinal Chemistry, The University of Utah, Salt Lake City, Utah
11/01-current Research Associate Professor, Department of Medicinal Chemistry, The University of Utah, Salt Lake City, Utah

Research Interests:

Synthetic chemistry
Synthesis of bacterial oxidosqualene cyclase inhibitors
Cancer immunotherapy
Targeted drugs
Design and synthesis of small molecule inhibitors of protein-protein signaling
Design and synthesis of fluorescent phosphoinositide probes
Research and technology management.

Honors:

Listed in "American Men and Women of Science"
Listed in "Who's Who in Technology"
Named Outstanding Senior in Chemistry, 1966
National Defense Education Act Title IV Fellow, 1968-1970
Franklin Award, Thiokol Corporation recognition for outstanding technical achievement, 1995

Publications/Patents: J. C. Hinshaw has over 50 publications and patents. A few are listed.

P. Y. Lum, C. D. Armour, S. B. Stepaniants, G. Cavet, A. Leonardson, P. Garrett-Engele, M. K. Wolf, L. Butler, C. M. Rush, M. Bard, J. C. Hinshaw, P. Garnier, G. D. Prestwich, G. Schimmack, J. W. Phillips, C. J. Roberts, and D. D. Shoemaker, "Discovering Novel Modes of Action for Therapeutic Compounds using a Genome-wide Screen of Yeast Heterozygotes," *Cell,* 2004, **116**, 121-137.

J. C. Hinshaw, D.-Y. Suh, P. Garnier, F. S. Buckner, R. T. Eastman, S. P. T. Matsuda, B. M. Joubert, I. Copper K. A. Joiner, S. Merali, T. E. Nash, and G. D. Prestwich, "Oxidosqualene Cyclase Inhibitors As Antimicrobial Agents," *J. Med. Chem.,* 2003, **46**, 4240.

T. M. McIntyre, A. V. Pontsler, A. R. Silva, A. St. Hilaire, Y. Xu, J. C. Hinshaw, G. A. Zimmerman, K. Hama, J. Aoki, H. Arai, and G. D. Prestwich, Identification of an intracellular receptor for lysophosphatidic acid (LPA): LPA is a transcellular PPARγ agonist, *Proc. Natl. Acad. Sci. USA,* 2003, **100**, 131.

S. Davies, A. Pontsler, G. Marathe, K. Harrison, R. Murphy, J. C. Hinshaw, G. D. Prestwich, A. Hilaire, S. Prescott, G. Zimmerman, and T. McIntyre, "Oxidized Alkyl Phospholipids are Specific, High Affinity PPARg Ligands", *J. Biol. Chem.*, 2001, **276**, 16015.

J. C. Hinshaw and G. D. Prestwich, "The Design, Synthesis, and Evaluation of Molecules That Enable or Enhance Cellular Uptake: Peptoid Molecular Transporters", *ChemTracts, Organic Chemistry*, 2001, **14**, 3[illegible] Commentary on the research by P. Wender, D. Mitchell, K. Pattabiraman, E. Pelkey, L. Steinman, and J. Rothbard, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 13003.

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R.B. Wardle, R.M. Hajik, J.C. Hinshaw, T.K. Highsmith, "Process for the Large-Scale Synthesis of 4,10-Dinitr 2,6,8,12-Tetraoxa-4,10-Diazatetracyclo[5.5.0.$0^{5,9}$$0^{3,11}$]dodecane," U.S. Patent 6,107,483, issued August 22, 2000.

J. C. Hinshaw, D. W. Doll, R. J. Blau, G. K. Lund, " Metal Complexes for Use as Gas Generants," U.S. Patent 6,039,820, issued March 21, 2000.

G. D. Prestwich, F. S. Buckner, J. C. Hinshaw, "Methods Related to Steroid Metabolism of Parasites and Mycobacteria, and Treatment of Parasite and Mycobacterial Infections with an Oxidosqualene Cyclase Inhibitor", U.S. Patent Application filed June 16, 2000.

R.B. Wardle, R.M. Hajik, J.C. Hinshaw, T.K. Highsmith, "Process for the Large-Scale Synthesis of 4,10-Dinitr 2,6,8,12-Tetraoxa-4,10-Diazatetracyclo[5.5.0.0]dodecane, "U.S. Patent 6,107,483, issued August 22, 2000.

J. C. Hinshaw, D. W. Doll, R. J. Blau, G. K. Lund, " Metal Complexes for Use as Gas Generants," U.S. Patent 6,039,820, issued March 21, 2000.

J. C. Hinshaw, D. W. Doll, R. J. Blau, G. K. Lund, "Metal Hydrazine Complexes Used as Gas Generants," U.S Patent 5,970,703, issued October 26, 1999.

J. C. Hinshaw, D. W. Doll, R. J. Blau, G. K. Lund, "Using Metal Complex Compositions As Gas Generants," U.S. Patent 5,735,118, issued April 7, 1998.

R. R. Bennett, J. C. Hinshaw, and M. Barnes, "The Effects of Chemical Propulsion on the Environment," *Acta Astronautica*, 26, 531 (1992).

J. C. Hinshaw and W. W. Edwards, "Synthesis of Tetranitropyrrole," *J. Hetercyclic Chem.*, 29, 1721 (1992).

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.

NAME	POSITION TITLE		
SHA, JIANG	Graduate Research Assistant		
EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.*)			
INSTITUTION AND LOCATION	DEGREE (*if applicable*)	YEAR(s)	FIELD OF STUDY
Peking University, Beijing, China	B.S.	1997-2001	Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

Research and Professional Experience:

2000 - 2001 Institution of Biophysics, Chinese Academy of Science

2001 - 2002 Molecular Biology Program, The University of Utah, Laboratory Rotation

2002 - current Graduate Research Assistant, Department of Medicinal Chemistry, The University of Utah, Salt Lake City

BIOGRAPHICAL SKETCH

Provide the following information for the Principal or Co-Principal Investigators
Follow this format for each person.

NAME	POSITION TITLE
HEE-KYOUNG LEE	Postdoctoral Research Associate

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing. Include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Seoul National University, Seoul, Korea	BS	1988	Chemistry
Seoul National University, Seoul, Korea	MS	1990	Biochemistry
Stony Brook University, New York, USA	Ph.D.	2003	Biochemistry and Cell Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

Education and Experience

1984-1988 B.S. in Chemistry, Seoul National University, Seoul, Korea
General Advisor: Professor Hasuck Kim
Research Topic: *Characterization of Subtilisin from Bacillus subtilis*

1988-1990 M.S. in Biochemistry, Seoul National University, Seoul, Korea
Research Advisor: Professor Chul-Hak Yang
Research Topic: *Cloning and Sequencing of Hydrogenase Gene from E. coli*

1990-1991 Full-time Teaching Assistant, Department of Chemistry, Seoul National University, Seoul, Korea

1991-1992 Full-time Teaching Assistant, Inter-University Instrument Facilities for Basic Science Research, Seoul National University, Seoul, Korea

1992-2003 Ph.D. in Biochemistry & Cell Biology, Stony Brook University, New York.
Research Advisor: Professor Glenn D. Prestwich
Research Topic: *Molecular Interactions in Squalene Epoxidase: Photoaffinity Labeling and Mutagenesis Studies*

Publications

Pamela Denner-Ancona, Mei Bai, Hee-Kyoung Lee, Ikuro Abe and Glenn D. Prestwich, "Purification of Pig and Rat Liver Squalene Epoxidase by Affinity Chromatography" *Bioorg. Med. Chem. Lett.*, **5**, 481-486 (1995)

Hee-Kyoung Lee and Glenn D. Prestwich, "Unusual Signaling Pathway of Steroid Hormones: Dual Action of Progesterone" *Chemtracts*, **12**, 40-44 (1999)

Hee-Kyoung Lee, Pamela Denner-Ancona, Jun Sakakibara, Teruo Ono and Glenn D. Prestwich, "Photoaffinity Labeling and Site-Directed Mutagenesis of Rat Squalene Epoxidase" *Arch. Biochem. Biophys.*, **381**, 43-52 (2000)

Hee-Kyoung Lee, Yi-Feng Zheng, Xiao-yi Xiao, Mei Bai, Jun Sakakibara, Teruo Ono and Gelnn D. Prestwich, "Identification of the Substrate Binding Site of Mammalian Squalene Epoxidase by Photoaffinity labeling with a Diazoacetate-Containing Substrate Analog" *J. Lipid Res.* (Submitted)